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METHOD AND MICROORGANISM FOR THE PRODUCTION OF D-MANNITOL

The invention relates to a method and the microorganism for the production of D-mannitol.

The world-wide consumption of the sugar alcohol D-mannitol is 30 000 tonnes per annum. D-mannitol is used in the foodstuffs industry as a sweetener which does not harm the teeth, in medicine as a plasma expander and vasodilator (hexanitro derivative), and in the pharmaceuticals industry in tablet production.

On a commercial scale, D-mannitol has so far been produced by the catalytic hydrogenation, over metal catalysts, of glucose/fructose mixtures as the starting materials. Because the catalytic hydrogenation is non-stereospecific, the yield of D-mannitol is only 25-30% with a threefold excess of D-sorbitol (Makkee M, Kieboom APG, Van Bekkum H (1985), Production methods of D-mannitol. *Starch/Stärke* **37**: 136-140).

D-mannitol and D-sorbitol differ only in their configuration at the C2 carbon atom (stereoisomers); separation of the undesirable sorbitol is therefore complex and associated with difficulties.

D- mannitol may alternatively be produced by enzymatic hydrogenation of D-fructose in a microbial biotransformation process, in which a recombinant mannitol dehydrogenase (MDH) is isolated from from *Pseudomonas fluorescens* and incubated together with a formiate dehydrogenase (FDH) from *Candida boidinii* and NADH in a membrane reactor (Slatner M et al. (1998) Biotransf. 16: 351-363). The use of the formiate dehydrogenase sets up a cycle of NADH reduction and oxidation, NADH being retained in the reaction vessel by the membrane. Only 70-90% of the fructose could be converted into D-mannitol in this way. Furthermore, the mannitol dehydrogenase used has poor stability

(half life: 50 h; after stabilisation with dithiothreitol: 100 h), sensitivity to high temperatures > 30 °C and to shearing forces. A further, major disadvantage is that membrane reactors are unsuitable for large-scale industrial production on account of the high cost of isolated enzymes, and the requisite co-factors and membranes.

A process of fermentation offers a further possible means for D-mannitol production, wherein yields of approximately 85% were obtained in a fermentation with growing cells using D-fructose/D-glucose mixtures as the substrate and the heterofermentative lactic-acid bacterium *Leuconostoc mesenteroides* ATCC 12291 as the catalysing organism (Soetaert (1991): Synthesis of D-mannitol and L-sorbose by microbial hydrogenation and dehydrogenation of monosaccharides. PhD Thesis, University of Gent)). The gene for MDH from *Leuconostoc pseudomesenteroides* and its characterisation are also known (J. Aarnikunnas et al., Applied Microbiology and Biotechnology, 13 July 2002). The reduction equivalents necessary for the reduction of fructose to D-mannitol originate from the oxidation of glucose to organic acids. Apart from the problem that the substrate fructose is only 85% converted to D-mannitol, the use of D-glucose is disadvantageous since contamination of the target substance with organic acids occurs during fermentation and these organic acids have to be removed by means of complex stages of the process. With fermentation with growing cells, 100% conversion of the substrate to the product can be achieved, since some of the substrate is used in cell construction or in the production of new biomass. The fermentation of *Leuconostoc mesenteroides* is furthermore difficult (formation of mucilage) and, owing to the complex media, expensive and the preparation of the supernatant is therefore also costly.

The literature discloses three further mannitol-2-dehydrogenases which, moreover, have been described in respect of their biochemical properties and nucleotide/amino acid sequences. These include the mannitol-2-dehydrogenase from *Pseudomonas fluorescens* DSM 50106 (Brünker P, Altenbuchner J, Mattes R (1998) Structure and function of the genes involved in mannitol, arabitol and glucitol utilisation from *Pseudomonas fluorescens* DSM 50106. *Gene* **206:** 117-126), from *Rhodobacter sphaeroides* Si4 (Schneider KH, Giffhorn F, Kaplan S (1993) Cloning, nucleotide sequence and

characterization of the mannitol dehydrogenase gene from *Rhodobacter sphaeroides*. J. Gen. Microbiology **139**: 2475-2484), and from *Agaricus bisporus* (Stoop JM, Mooibroeck H (1998) Cloning and characterization of NADP-mannitol dehydrogenase cDNA from the button mushroom *Agaricus bisporus*, and its expression in response to NaCl stress. *Appl. Environ. Microbiol.* **64**: 4689-4696.). The first two belong to the long-chain dehydrogenase/reductase (LDR) protein family, and the last to the short-chain dehydrogenase/ reductase (SDR) protein family.

The aim of the invention is therefore to provide an improved method for the production of D-mannitol which avoids the described disadvantages of the prior art.

The aim is achieved with a method of producing D-mannitol with the use of a mannitol-2-dehydrogenase (MDH)-expressing organism, wherein the sugar substrates and/or sugar substrate precursors of MDH are transported into the organism via a non-phosphorylating sugar transport system.

The aim is achieved with the method according to the invention in that the sugar to be converted with MDH can be converted to D-mannitol by the MDH directly without prior phosphorylation. Surprisingly, this direct conversion enables yields and concentrations of D-mannitol in the reaction supernatant to be improved by comparison with the prior art to date. In some cases, yields of up to 100% with reference to the substrate (glucose) are already possible with the method according to the invention. Furthermore, concentrations of up to 40 g/L are obtained with the method of the invention.

The term organism is understood to mean both single-celled and multicellular organisms, in particular microorganisms.

The aim is further achieved with a microorganism which expresses the enzymes MDH according to sequence No. 2 and FDH according to sequence No. 3 for the microbial production of D-mannitol and has a non-phosphorylating sugar transport system which

transports the sugar substrate and/or sugar substrate precursors of MDH into the micro-organism.

The designation D-mannitol should be understood as also referring to D-mannit.

Within the scope of this invention, all nucleotide sequences coding for a mannitol-2-dehydrogenase will be grouped together under the designation "*mdh*-gene sequence". The enzyme mannitol-2-dehydrogenase will, in what follows, be designated "MDH".

Correspondingly, all nucleotide sequences coding for a formiate dehydrogenase will be grouped together under the designation "*fdh*-gene sequence". The enzyme formiate dehydrogenase will, in what follows, be designated "FDH".

Furthermore, all nucleotide sequences coding for a glucose facilitator will be grouped together under the designation "*glf*-gene sequence". The protein glucose facilitator will, in what follows, be designated "GLF".

The term nucleotide sequence will furthermore be understood to include all nucleotide sequences which (i) correspond exactly to the represented sequences; or (ii) comprise at least one nucleotide sequence which corresponds to the represented sequences within the range of degeneracy of the genetic code; or (iii) comprise at least one nucleotide sequence which hybridises with a nucleotide sequence complementary to nucleotide sequence i) or ii), and optionally (iiii) comprise functionally-neutral sense mutations in (i). Here, the term functionally-neutral sense mutations means the exchange of chemically similar amino acids, e.g. glycine with alanine, or serine with threonine.

The sequence regions preceding (5' or upstream) and/or following (3' or downstream) the coding regions (structural genes) are also included according to the invention. Sequence regions having regulatory function are included in particular. These may influence transcription, RNA stability or RNA processing, as well as translation. Examples of

regulatory sequences are, inter alia, promoters, enhancers, operators, terminators or translation enhancers.

The respective enzymes also include isoforms, which are understood to be enzymes having identical or comparable substrate specificity and specificity of action, but which have differing primary structures.

Modified forms according to the invention are understood as enzymes in which variations are present in the sequence, for example at the N-end and/or C-end of the polypeptide or in the region of conserved amino acids, without, however, impairing the function of the enzyme. These modifications may be made by known methods in the form of amino-acid substitutions.

The sugar transport system is advantageously the glucose facilitator (GLF) according to nucleotide sequence No. 1, which preferably originates from a eukaryote e.g. from a yeast. The GLF from Zymomonas mobilis, which was cloned by T. Conway et al. (Journal of Bacteriology, Dec. 1990, pp. 7227-7240) may also be preferably used.

German patent application 198 18 541.3 admittedly discloses a method for the production of substances from the aromatic metabolism using a microorganism which shows an elevated activity of a glucose-oxidising enzyme and converts glucose or glucose-containing substrates by oxidation to gluconolactone and gluconate and, by phosphorylation of the gluconate, to 6-phosphogluconate wherein, in addition to the increased enzyme activity of the oxidase and/or of the phosphatase to increase the quantity of PEP present, the activity of a PEP-independent glucose transport protein is increased, which may be the glucose facilitator (GLF) from Zymomonas mobilis. However, no method for producing mannitol may be inferred therefrom.

As well as fructose, the GLF may also transport glucose or xylose, glucose in particular being interesting as a cost-effective fructose precursor. The glucose may be converted into fructose, as will be described below.

The MDH-coding sequence from microorganisms from the Lactobacteriaceae family, especially preferably from Leuconostoc pseudomesenteroides, is especially suitable for the conversion to D-mannitol owing to the high activity and stability of the MDH synthesised therefrom.

The organism preferably expresses sequence No. 2 coding for MDH.

Microorganisms from the genus Bacillus, Pseudomonas, Lactobacillus, Leuconostoc, the Enterobacteriaceae or methylotrophic yeasts and fungi are especially suitable as the organism. All microorganisms used in the foodstuffs industry may also be used.

The organism used originates especially preferably from the group: Achromobacter parvolus, Methylobacterium organophilum, Mycobacterium formicum, Pseudomonas spec. 101, Pseudomonas oxalaticus, Moraxella sp., Agrobacterium sp., Paracoccus sp., Ancylobacter aquaticus, Maxcobacterium [sic; recte: Mycobacterium] vaccae, Pseudomonas fluorescens, Rhodobacter sphaeroides, Rhodobacter capsulatus, Lactobacillus sp., Lactobacillus brevis, Leuconostoc pseudomesenteroides, Gluconobacter oxydans, Candida boidinii, Candida methylica, or even Hansenula polymorpha, Aspergillus nidulans or Neurospora crassa, or in particular Escherichia coli or Bacillus subtilis.

Analysis of the D-mannitol concentration may be carried out enzymatically/photometrically by K. Horikoshi's method (Horikoshi K. (1963) Meth. Enzym. Analysis, 3rd ed; vol. 6. H.U. Bergmeyer, ed., Verlag Chemie, Weinheim), or by high-pressure liquid chromatography (HPLC), as described by Lindroth et al. (Lindroth et al. (1979) Analytical Chemistry 51: 1167-1174).

A sequence coding for formiate dehydrogenase (FDH) may be used to set up an oxidation-reduction cycle. It preferably originates from Mycobacterium vaccae and has a nucleotide sequence according to sequence No. 3. This is known per se from K. Soda et al., Appl. Microbiol. Biotechnol. (1995) 44, 479-483.

By this means, a considerable increase in the yield or reaction rate of the substrate fructose into mannitol is made possible by creating a cofactor-regeneration system. In this arrangement, the substrate is no longer consumed in the preparation of the reduction equivalents necessary for the reduction of fructose to mannitol, rather they are prepared by a second enzyme system. Consequently, more of the coenzyme NADH is available for conversion to mannitol. One of the most commonly used systems is regeneration with a formiate dehydrogenase, e.g. from Mycobacterium vaccae. Through the use of this enzyme, together with an arbitrary MDH, preferably from Leuconostoc pseudomesenteroides, an oxidation-reduction cycle is set up in which formiate functions as an electron donor and D-fructose as an electron acceptor. Here, the enzyme formiate dehydrogenase catalyses the oxidation of formiate to CO_2 and the enzyme MDH the reduction of D-fructose to D-mannitol (see Fig. 1). The intracellular nicotinic acid amide-adenine-dinucleotide (NAD) pool serves as an electron shuttle between the two enzymes. The oxidation of formiate to CO_2 is thermodynamically advantageous, since the standard free energy of formation ΔG^o for CO_2 is distinctly negative and the CO_2 is removed from the reaction equilibrium by gaseous escape. The increased intracellular NADH concentration resulting, inter alia, from formiate oxidation increases the reductive power for the reduction of D-fructose to D-mannitol, catalysed by MDH.

In a further advantageous embodiment of the method, in addition to the carbon sources already mentioned D-glucose is used as a substrate in the production. D-glucose may be converted to D-fructose with the enzyme D-glucose/xylose isomerase (EC 5.3.1.5) (2). Both intracellular and extracellular conversion are possible. The use of D-glucose as a substrate in a method of producing D-mannitol clearly improves the cost-effectiveness of the method.

Microorganisms suitable for the method described include, not only ones into which a formiate dehydrogenase and an MDH are inserted and/or enhanced, but also microorganisms which already possess a formiate dehydrogenase or optionally an MDH, e.g. Achromobacter parvolus, Methylobacterium organophilum, Mycobacterium formicum, Pseudomonas spec. 101, Pseudomonas oxalaticus, Moraxella sp., Agrobacterium sp.,

Paracoccus sp., Ancylobacter aquaticus. These include microorganisms such as Pseudomonas fluorescens, Rhodobacter sphaeroides, Rhodobacter capsulatus, Lactobacillus sp., Lactobacillus brevis, Gluconobacter oxydans and preferably also Leuconostoc pseudomesenteroides, or microorganisms already possessing both enzymes, the activity of which is enhanced in each case. Also suitable are, for example, methylotrophic yeasts such as Candida boidinii, Candida methylica, or even Hansenula polymorpha, fungi such as Aspergillus nidulans and Neurospora crassa, as well as all microorganisms also used in the foodstuffs industry.

With the method according to the invention and the microorganisms, it is now possible to achieve improved conversion of the substrate into the substance D-mannitol. By comparison with previously known methods, increased productivity and an increased yield of D-mannitol (up to 100%) are achieved. Concentrations of up to 40 g/L have already been achieved. The method is therefore especially suitable for the profitable production of D-mannitol on a large industrial scale. Via creation of the regeneration system with the aid of formiate dehydrogenase, increased conversion of the substrate into the product D-mannitol is made possible for NADH-consuming MDH with resting cells, to an increased degree without the disadvantageous formation of metabolic by-products.

The invention also includes the use of nucleotide sequences according to sequences Nos. 1, 2 and 3 coding for GLF, MDH and FDH for use in any one of the above microorganisms.

The invention also includes a gene structure containing at least one or more of the above nucleotide sequences.

A vector containing one or more of the above nucleotide sequences or one or more of the aforementioned gene structures also falls within the scope of the invention.

The invention also includes the use of the aforementioned nucleotide sequences, gene structures and vectors in the described microorganisms or in microorganisms containing these nucleotide sequences, gene structures and vectors.

The drawings show, by way of example, results of the method according to the invention as well as a schematic representation of the most important metabolic pathways which play a role in the method.

The Figures are as follows:

Fig. 1: Schematic representation inside a cell, of a redox cycle with formiate dehydrogenase and MDH;

Fig. 2: Derivation of a degenerate 24-base oligonucleotide probe from the N-terminal amino acid sequence of the MDH subunit of *Leuconostoc pseudomesenteroides* ATCC 12291;

Fig. 3: Gene chart of the 4,191 bp Eco RI fragment isolated from the genomic DNA-plasmid bank of Leuconostoc pseudomesenteroides ATCC 12291 following immunoscreening of the *mdh* gene. The arrows indicate the direction of translation of the *mdh* ORF and 4 ORFs.

Sequence No. 1 shows the nucleotide sequence coding for GLF from Zymomonas mobilis.
Sequence No. 2 shows the nucleotide sequence coding for MDH from Leuconostoc pseudomesenteroides.
Sequence No. 3 shows the nucleotide sequence coding for FDH from Mycobacterium vaccae N10.

In what follows the invention will be described with the use of examples.

I) Mannitol-2- dehydrogenase from Leuconostoc pseudomesenteroides ATCC 12291: purification and characterisation of the enzyme; cloning and functional expression of the *mdh* gene in Escherichia coli

a) Bacterial strains and plasmids

Leuconostoc pseudomesenteroides ATCC 12291 was used as the source for isolation of MDH. *E. coli* JM109 (DE 3) (Promega) served as the host organism for production of a partial plasmid bank for isolation of the genomic DNA from *Leuconostoc pseudomesenteroides* ATCC 12291. Part of the plasmid bank was produced in pUC18, by ligation of a 4.0 – 4.5 kb Eco RI fragment of genomic DNA from *Leuconostoc pseudomesenteroides* ATCC 12291.

b) Culturing conditions

The following culture medium was used for culturing Leuconostoc pseudomesenteroides ATCC 12291:

Trypton 10 g/l, yeast extract 10 g/l, K_2HPO_4 10 g/l, D-fructose 20 g/l, D-glucose 10 g/l, vitamin/mineral solution 10 ml/l, in distilled water; pH adjusted to 7.5 with the use of ortho-phosphoric acid.

For subcloning and preparation of plasmid bank of the genomic *Leuconostoc* DNA, *E. coli* JM109 (DE 3) was cultured at 170 rpm and 37 °C in Luria-Bertani medium with addition of ampicillin (100 μg/ml) or carbenicillin (50 μg/ml).

c) Determination of the activity of MDH from Leuconostoc pseudomesenteroides ATCC 12291

In the present invention, the enzyme activity is determined photometrically via the decrease in the NADH concentration for the reduction reaction

$$\text{D-fructose} + \text{NADH} + \text{H}^+ \rightarrow \text{D-mannitol} + \text{NAD}^+.$$

The batch for measuring the activity of the MDH contained 200 µM NADH and 200 mM D-fructose in 100 mM potassium phosphate buffer at pH 6.5. The specific activities of the raw extracts and partially purified enzyme isolates are given as units per milligram of protein (U/mg), 1 U being defined as 1 µmol of substrate decrease per minute.

d) Determination of the protein concentrations

All protein concentration determinations were performed using Bradford's method.

e) Separation of proteins by polyacrylamide gel electrophoresis

Purity analyses of raw extracts and partially purified enzyme isolates, and preparations preparatively on Western blots were carried out by electrophoresis in discontinuous 12% SDS-polyacrylamide gels using Lämmli's method.

f) Isolation of mannitol-2-dehydrogenase from *Leuconostoc pseudomesenteroides* ATCC 12291

To isolate the mannitol-2-dehydrogenase, after cellular disintegration, the following process steps were carried out: ammonium sulfate precipitation, hydrophobic interaction chromatography, anion exchange chromatography I, anion exchange chromatography II, size exclusion chromatography, and chromatofocusing pH 5 – 4.

At pH = 5.35 , the specific activity of the MDH for the reduction of D-fructose to D-mannitol was 450 U/mg.

g) Molecular-genetic methods

The isolation of genomic DNA from *Leuconostoc pseudomesenteroides* ATCC 12291, the isolation of DNA fragments from agarose gels, the labelling of DNA probes with digoxigenin-modified dUTP, and immunological detection and DNA-DNA hybridisation (Southern blot) were carried out.

The aminoterminal sequencing of the 43 kDa-enzyme subunit by means of Edman degradation and subsequent HPLC analysis yielded the octameric amino-acid sequence MEALVLTG. Using codon usage statistics for *Leuconostoc pseudomesenteroides*, a 2048-fold degenerate oligonucleotide probe for detection of the mannitol-2-dehydrogen-ase gene in genomic DNA of *Leuconostoc pseudomesenteroides* ATCC 12291 was derived (see Fig. 2). The 24 bp-DNA probe was provided with a digoxenin-11-dUTP tail at the 3' end and served for the immunoscreening of partial plasmid banks of genomic DNA from L. pseudomesenteroides ATCC 12291. By this pathway, a 4.2 kb DNA fragment was isolated (Fig. 3). With suitable primers, the *mdh* gene from this fragment was amplified, ligated into the vector pET24a(+), and transformed and expressed in E. coli BL21 (DE 3). Cell extracts from E. coli BL21 (DE 3) pET24a (+) L*mdh* showed, following induction in SDS-polyacrylamide gel electrophoresis, a pronounced over-expression band at 43 kDa and a specific activity of the mannitol-2-dehydrogenase of 70 U/mg of protein, whereas the controls (cells without plasmid, cells with blank plasmid) showed no activity.

The nucleotide sequence of the *mdh* gene from L. pseudomesenteroides ATCC 12291 is shown in sequence No. 2.

II) Biotransformation of D-fructose to D-mannitol with a recombinant E. coli strain

In a recombinant E. coli strain, the enzymes formiate dehydrogenase (EC 1.2.1.2) and mannitol-2-dehydrogenase (EC 1.1.1.67) were overexpressed in order to establish an oxidation-reduction cycle in the cells. In this oxidation-reduction cycle, hydrogen is

transferred from formiate via cellular NAD^+ to D-fructose, during which D-fructose is reduced to give D-mannitol (see Fig. 1). The glucose facilitator was additionally expressed in the cells, in order to improve the availability of the substrate fructose.

(a) Strains and vectors

Strains of E. coli BL21 (DE 3) Star (Invitrogen) were used. pET-24a (+) *fdh/mdh* coding for the ORF of formiate dehydrogenase from Mycobacterium vaccae and of mannitol-2-dehydrogenase from Leuconostoc pseudomesenteroides, and pZY507 *glf* coding for the glucose facilitator from Zymomonas mobilis were used as vectors.

For the biotransformation, chemically competent E. coli BL21 (DE 3) Star was cotransformed with pET-24a (+) *fdh/mdh* and pZY507 *glf* and selected on LB-agar plates with 25 µg/ml of chloramphenicol and 30 µg/ml of kanamycin. As the controls, E. coli BL21 (DE 3) Star was transformed either with pET-24a (+) *fdh/mdh* or with pZY507 *glf* alone. Selection of the transformands was performed on LB-agar plates with either 25 µg/ml of chloramphenicol (pZY507 *glf*) or 50 µg/ml of kanamycin (pET-24a (+) *fdh/mdh*). LB-agar plates for E. coli BL21 (DE 3) Star transformed with pET-24a (+) *fdh/mdh* additionally contained 1% (v/v) D-glucose to reduce the basal expression of mannitol-2-dehydrogenase and formiate dehydrogenase.

(b) Biotransformation

Following the overexpression of FDH, MDH and GLF in E. coli, non-growing cells were used in a biotransformation. Portions of 1.0 g of induced cells of E. coli BL21 (DE 3) Star pET-24a (+) *fdh/mdh* / pZY507 *glf* were washed with 100 mM potassium phosphate buffer of pH 7.0 and re-suspended in 50 ml of reaction solution with 500 mM of D-fructose and 500 mM of sodium formiate in 100 mM of potassium-phosphate buffer of pH 6.7. The batches were agitated in 100-ml unbaffled flasks at 100-120 rpm and 30 °C for 24 h. 1-2 ml samples of the supernatant were withdrawn at the times 0, 1, 2, 3, 4, 5, 6, 8, 17 and 23 h after the start of the reaction for measurement of the concentrations of

formiate, D-fructose and D-mannitol. The samples were centrifuged at 5000 g for 1 min, the supernatant was 0.2 μm-filtered and stored for HPLC measurement at -20 °C. As a control, 1.0 g of non-induced cells of E. coli BL21 (DE 3) Star pET-24a (+) *fdh*/*mdh* / pZY507 *glf* were used in the biotransformation in the same way.

The concentration determinations of formiate, D-fructose and D-mannitol in the reaction supernatant and in the cell-free raw extract were carried out using an HPLC system (Merck/Hitachi).

Table 1 shows, by way of example, results achieved with transformed microorganisms.

It was demonstrated that the parallel overexpression of formiate dehydrogenase, mannitol-2-dehydrogenase and of the glucose facilitator in E. coli leads to a very high production of D-mannitol by these cells in a reaction medium with D-fructose and formiate. The up to 244 mM of mannitol after 23 h are to be compared with the method without GLF (only 15 mM after 17 h) and the method without FDH (20 mM after 17 h). An approximately 12-15 fold improvement was therefore achieved.

Table 1:

Recombinant genes	Conditions	Mannitol production [mM]	Fructose consumption [mM]	Formiate consumption [mM]
fdh / mdh/ glf	+ IPTG/ + formiate / 23 h	244	332	471
fdh / mdh/ glf	- IPTG/ + formiate / 17 h	0	n.d.	n.d.
fdh / mdh/ glf	+ IPTG/ - formiate / 17 h	11	76	n.d.
mdh/ glf	+ IPTG/ + formiate / 17 h	20	48	81
mdh/ glf	- IPTG/ + formiate / 17 h	0	n.d.	n.d.
fdh /mdh	+ IPTG/ + formiate / 17 h	15	61	159
fdh /mdh	- IPTG/ + formiate / 17 h	0	n.d.	n.d.
mdh	+ IPTG/ + formiate / 17 h	0	57	35
mdh	- IPTG/ + formiate / 17 h	0	n.d.	n.d.
fdh / glf	+/- IPTG/ + formiate / 17 h	0	n.d.	n.d.
fdh	+/- IPTG/ + formiate / 17 h	0	n.d.	n.d.
glf	+/- IPTG/ + formiate / 17 h	0	n.d.	n.d.